Use of a Hierarchical Oligonucleotide Primer Extension Approach for Multiplexed Relative Abundance Analysis of Methanogens in Anaerobic Digestion Systems

Jer-Horng Wu, Hui-Ping Chuang, Mao-Hsuan Hsu, Wei-Yu Chen

Department of Environmental Engineering, National Cheng Kung University, Tainan City, Taiwan, Republic of China

In this study, we established a rapid multiplex method to detect the relative abundances of amplified 16S rRNA genes from known cultivatable methanogens at hierarchical specificities in anaerobic digestion systems treating industrial wastewater and sewage sludge. The method was based on the hierarchical oligonucleotide primer extension (HOPE) technique and combined with a set of 27 primers designed to target the total archaeal populations and methanogens from 22 genera within 4 taxonomic orders. After optimization for their specificities and detection sensitivity under the conditions of multiple single-nucleotide primer extension reactions, the HOPE approach was applied to analyze the methanogens in 19 consortium samples from 7 anaerobic treatment systems (i.e., 513 reactions). Among the samples, the methanogen populations detected with order-level primers accounted for >77.2% of the PCR-amplified 16S rRNA genes detected using an *Archaea*-specific primer. The archaeal communities typically consisted of 2 to 7 known methanogen genera within the *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* and displayed population dynamic and spatial distributions in anaerobic reactor operations. Principal component analysis of the HOPE data further showed that the methanogen communities could be clustered into 3 distinctive groups, in accordance with the distribution of the *Methanosaeta*, *Methanolinea*, and *Methanomethylovorans*, respectively. This finding suggested that in addition to acetotrophic and hydrogenotrophic methanogens, the methylotrophic methanogens might play a key role in the anaerobic treatment of industrial wastewater. Overall, the results demonstrated that the HOPE approach is a specific, rapid, and multiplexing platform to determine the relative abundances of targeted methanogens in PCR-amplified 16S rRNA gene products.

The anaerobic digestion process has become a method increasingly used for the recycling of organic substances in waste and wastewater into methane as a renewable energy source (1). The process can be operated using different reactor formats under specific environmental conditions with various microbial groups involved in a multistep reaction. Because of their key position in the terminal step that produces the gaseous final products methane and carbon dioxide, the reactor performance ultimately relies on the activities of the methanogen population. Therefore, increased understanding of the relevant population structures and their ecological significance inside the reactor could provide key information with which to increase the efficiency of organic matter decomposition and methane recovery and achieve better reactor output.

The methanogenic archaea (methanogens) are a group of strict anaerobes that use a limited number of substrates, such as H_2/CO_2 (hydrogenotrophic methanogens), acetate (acetotrophic methanogens), and methyl group-containing compounds (methylotrophic methanogens) (2). They can be detected through the analysis of their unique cellular features (3, 4), as well as, more effectively, analysis using molecular biological tools (5). For example, to reveal the structure of methanogen populations in environmental samples, the community DNA is first recovered. Then, the nucleotide sequences of the 16S rRNA gene or the gene coding for the α subunit of methyl coenzyme M reductase (*mcrA*) relevant to methanogens is PCR amplified and analyzed with molecular techniques, such as cloning and sequencing (6), denaturing gradient gel electrophoresis (DGGE) (7), terminal restriction fragment length polymorphism (8), single-stranded conformation polymorphism (SSCP) (9), amplicon length PCR (10), or a

combination of these. Alternatively, hybridization-based methods, such as membrane hybridization (11, 12) and fluorescence *in situ* hybridization (FISH) (13), and the quantitative PCR (qPCR) method (14, 15) combined with oligonucleotide primers/probes specifically targeting the selected methanogen groups can be used for quantitative analysis. Narihiro and Sekiguchi recently reported a comprehensive set of probes targeting the methanogens at various taxonomic specificities (16), enabling the rapid evaluation of the relative abundances of the methanogen populations at different hierarchies (17). However, because of limitations in their throughput capabilities, the implementation of the described methods requires a large number of primers/probes and tends to be laborious and time-consuming. Therefore, a method that facilitates the high-throughput determination of the relative abundance of methanogens in the complex microbial consortia is desired.

In a previous study, we developed the hierarchical oligonucleotide primer extension (HOPE) method for the rapid and multiplexed analysis of the relative abundance of multiple microbial targets in PCR amplicons from complex environmental samples

Received 21 July 2013 Accepted 23 September 2013

Published ahead of print 27 September 2013

Address correspondence to Jer-Horng Wu, enewujh@mail.ncku.edu.tw.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.02450-13>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.02450-13

TABLE 1 Methanogenic consortia analyzed by the HOPE method in this study

Sample no.	Biomass format	Reactor		Feeding substrate ^b	Temp ^c (°C)	Organic loading ^d	% removal
		Type ^a	Scale (sampling ht or date)				
1	Granule	UASB	Full scale (1 m)	PTA WW	Ambient	2.26 g TOC/liter	90
2	Granule	UASB	Full scale (1 m)	PTA WW	Ambient	2.26 g TOC/liter	71
3	Biofilm	FF	Lab scale	Terephthalate	50	4.0 g TA/liter	90–95
4	Granule	FB	Lab scale	<i>p</i> -Toluate	35	0.3 g <i>p</i> -Tol/liter	>95
5	Biofilm	AFB	Full scale (3 m)	Phenolic resin WW	Ambient	2.5–3.0 g COD/liter	70–85
6	Biofilm	AFB	Full scale (19 m)	Phenolic resin WW	Ambient	2.5–3.0 g COD/liter	70–85
7	Granule	UASB	Full scale (1 m)	Pulp and mill WW	Ambient	NA ^e	NA
8	Sludge	UASB	Full scale (0.3 m)	TFT-LCD WW	Ambient	0.2–0.3 g TOC/liter	85–90
9	Sludge	UASB	Full scale (1 m)	TFT-LCD WW	Ambient	0.2–0.3 g TOC/liter	85–90
10	Sludge	UASB	Full scale (2 m)	TFT-LCD WW	Ambient	0.2–0.3 g TOC/liter	85–90
11	Sludge	UASB	Full scale (3 m)	TFT-LCD WW	Ambient	0.2–0.3 g TOC/liter	85–90
12	Sludge	UASB	Full scale (4 m)	TFT-LCD WW	Ambient	0.2–0.3 g TOC/liter	85–90
13	Sludge	AD	Full scale (Sept. 2009)	WAS	Ambient	1 kg SS/m ³ /day	NA
14	Sludge	AD	Full scale (Oct. 2009)	WAS	Ambient	1 kg SS/m ³ /day	NA
15	Sludge	AD	Full scale (Jan. 2010)	WAS	Ambient	1 kg SS/m ³ /day	NA
16	Sludge	AD	Full scale (Oct. 2009)	WAS	Ambient	0.5–0.8 kg SS/m ³ /day	NA
17	Sludge	AD	Full scale (Nov. 2009)	WAS	Ambient	0.5–0.8 kg SS/m ³ /day	NA
18	Sludge	AD	Full scale (Dec. 2009)	WAS	Ambient	0.5–0.8 kg SS/m ³ /day	NA
19	Sludge	AD	Full scale (Jan. 2010)	WAS	Ambient	0.5–0.8 kg SS/m ³ /day	NA

^a UASB, up-flow anaerobic sludge bed reactor; FF, fixed film reactor; FB, fed-batch reactor; AFB, anaerobic fluidized bed reactor; AD, anaerobic digester.

^b PTA WW, purified terephthalic acid manufacturing wastewater; WW, wastewater; TFT-LCD WW, thin-film transistor liquid crystal display manufacturing wastewater; WAS, waste-activated sludge.

^c Ambient temperature is 26 to 32°C.

^d TOC, total organic carbon; COD, chemical oxygen demand; TA, terephthalate; *p*-Tol, *p*-toluate; SS, suspended solid.

^e NA, not available.

(18). This technique arranges multiple oligonucleotide primers that target the regions of the 16S rRNA genes at various phylogenetic specificities in a single-nucleotide strand extension reaction, allowing the DNA polymerase to incorporate a single fluorescently labeled dideoxynucleotide triphosphate (e.g., the dye terminators) at the 3' ends of the primers. An automatic DNA sequencer then analyzes the size and color of the extended primers. According to the hierarchical design concept, the results from the correctly extended primers provide phylogenetic information on the detected targets. The relative abundance of the targeted group relative to its higher taxonomic rank can also be determined according to the ratios of the fluorescence intensities obtained using 2 primers that have an associated hierarchical relationship. The HOPE method can achieve single-mismatch discrimination and high multiplex capabilities. With preamplification of the 16S rRNA gene by PCR, HOPE reached an excellent detection sensitivity (18) and has been applied to the study of the *Bacteroides* and *Prevotella* groups within the *Bacteroidales* (19, 20), cyanobacteria (21), and *Dehalococcoides* (22).

In this study, we constructed a set of 27 16S rRNA-based primers with specificities spanning the domain, order, and genus levels for methanogens and combined them using the HOPE approach. After optimizing the method with reference strains, we used the developed HOPE technique to evaluate the relative abundances of methanogens inside the anaerobic digestion systems treating industrial wastewater and sewage sludge. By using this approach, we were able to provide insights into the structure, dynamics, and distributions of the methanogen populations among the evaluated microbial consortia and successfully identify the key groups associated with different anaerobic treatment systems. These findings could facilitate the development of improved methods for the

management of methane production in anaerobic treatment systems.

MATERIALS AND METHODS

Methanogen reference strains. Twenty-four methanogen cultures obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany) or the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan) were used as the source of reference organisms (see Fig. S1 in the supplemental material). A *Methanocella paludicola* culture (strain SANAE) was kindly provided by Hiroyuki Imachi.

Environmental samples. In this study, we analyzed 19 consortium samples collected from different types of reactors and wastewater, sampling heights in a reactor, or sampling times during reactor operations using the HOPE method. As shown in Table 1, granule samples 1 and 2 were collected from full-scale up-flow anaerobic sludge bed (UASB) reactors treating purified terephthalic acid (PTA) wastewater, in which acetate, benzoate, terephthalate, and *p*-toluate account for the major components in the wastewater stream (23). The terephthalate-degrading biofilm sample (sample 3) was taken from a thermophilic (50°C) fixed film reactor (24), and the *p*-toluate-degrading granule sample (sample 4) was obtained from a mesophilic (35°C) fed-batch reactor in the laboratory. Both reactors inoculated with sludge sample 2 were operated for at least 6 months. In a 20-m-high full-scale anaerobic fluidized bed (AFB) reactor treating phenolic resin wastewater (25), 2 samples that were biofilm attached on granular activated carbon were obtained from sampling ports at 3-m and 19-m heights (samples 5 and 6, respectively). Sludge sample 7 was taken from a full-scale UASB reactor treating pulp and mill wastewater. Five sludge samples (samples 8 to 12) were taken from the sampling ports at different heights (0.3 m, 1 m, 2 m, 3 m, and 4 m, respectively) of a full-scale UASB reactor treating thin-film transistor liquid crystal display (TFT-LCD) manufacturing wastewater, in which tetramethyl ammonium hydroxide constituted the main wastewater ingredient (26). Sam-

ples 13 to 15 and samples 16 to 19 were obtained from 2 different full-scale anaerobic digesters for the treatment of waste sludge in a sewage treatment plant from September 2009 to January 2010. Samples were collected and stored at -80°C prior to analysis.

DNA preparation and PCR amplification of the archaeal 16S rRNA sequence. Total DNA from reference cultures and environmental samples was prepared using different methods. In brief, to quickly obtain DNA templates from reference cultures, the methanogen cells were harvested using centrifugation, washed with sterile Milli-Q water, and resuspended in a buffer solution containing 0.5% Triton X-100, 5 mmol/liter dithiothreitol, and 500 mmol/liter Tris-HCl (pH 7.5). Genomic DNA was prepared after disrupting the cell suspension using a microwave irradiation treatment, as described previously (27). For the recovery of DNA from various microbes in environmental samples, the microbial cells were subjected to freeze-thaw, enzymatic, and chemical treatments, and their community DNAs were purified and precipitated using phenol-chloroform-isomyl alcohol and ethanol precipitation procedures (28), followed by cleanup with a Wizard DNA cleanup kit (Promega, Madison, WI). The archaeal 16S rRNA gene fragments were obtained by PCR amplification with forward primer A1F (29) or A109F and reverse primer 1492R (30). Approximately 5 to 50 ng genomic DNA was used in a 100- μl PCR solution and subjected to thermocycling: 95°C for 5 min; 20 to 25 cycles of DNA denaturation at 95°C for 45 s, annealing at 52°C for 30 s, and primer extension at 72°C for 45 s; and a final extension at 72°C for 5 min in a thermocycler (Biometra, Germany). The amplicons were concentrated and purified using a QIAquick PCR purification kit (Qiagen), and their concentrations were quantified using a NanoVue spectrophotometer (Fisher Scientific, Uppsala, Sweden). Partial 16S rRNA sequences were obtained for validating the purity of the reference methanogens.

Design and arrangement of hierarchical primers. The 16S rRNA-targeted oligonucleotide primers (Table 2) were newly designed or modified from primers/probes for methanogens reported previously. These primers were assessed *in silico* according to key criteria, including primer characteristics (direction, extended nucleotides, length, GC content, and melting temperature) and the number and position of the mismatch that occurred in the nontargets from the study by Wu et al. (31) using ARB software (32) and an aligned 16S rRNA sequence database (SSU Ref version 96; <http://www.arb-silva.de>) that was updated with 420 almost-full-length archaeal sequences ($\sim 1,400$ nucleotides [nt]) reported from 2009 to 2011. The stabilities of the primer-template duplex and other possible duplex structures (hairpin, self-dimer, and heterodimer) were evaluated using the tools provided with OligoAnalyzer version 3.1 software (Integrated DNA Technologies). Multiple primers were arranged in a single tube on the basis of the phylogenetic affiliations of the methanogen targets (Table 2 and Fig. 1), so that the relative abundance of the individual archaeal target detected by each specific primer relative to the abundances of the targets detected by higher-level primers could be determined. The melting temperature and the labeled dye terminator (color) of the primers were also considered to ensure consistency in the fluorescence signals among the primers in a tube. To clearly differentiate extended primers in the same HOPE reaction mixture, primers that were extended with the same dye terminator were modified using different lengths of tails at their 5' ends.

HOPE analysis. For HOPE analysis, a single-nucleotide primer extension reaction mixture was prepared in a 5- μl solution containing 2.5 μl of the ready-to-use premix from a SNaPshot multiplex kit (Applied Biosystems), 5 to 20 ng purified DNA amplicons, and 1.5 μM (each) the unlabeled oligonucleotide primers (Mission Biotech, Taiwan). The reaction was conducted in a thermocycler (Biometra, Germany) with 20 cycles of 96°C for 10 s, 62°C for 20 s, and 72°C for 15 s. The 3' ends of the primers were extended with a single dye terminator upon the primer-template duplex formed, because the premix contained AmpliTaq DNA polymerase, salt, and buffer. The resulting products were then treated with shrimp alkaline phosphatase (Promega, Germany) at 37°C to minimize the possible background fluorescence caused by unincorporated dye terminators

(21), prior to their subjection to capillary electrophoresis with a GeneScan Liz 120 standard using an ABI Prism 3130 genetic analyzer (Applied Biosystems). The electrophoretic program included a denaturation step (60°C), an injection step in which a voltage of 2.1 kV was applied for 40 s, and a separation step. The electrophoretic size and fluorescent intensity of each of the extended primers were recorded for calculation of calibration factors and the relative abundances of the methanogen populations, as described previously (20). To ensure data reproducibility, each sample was analyzed in triplicate.

PCA. A principal component analysis (PCA) was used to evaluate the structural similarities among the methanogen populations quantified in the samples. Prior to the PCA, the relative abundances of methanogens detected using genus-specific primers were expressed as the percentages of the total archaeal population detected using an *Archaea*-specific primer. The data matrix (19 cases $\times$ 11 variables), based on the covariances, was analyzed using Statistica version 8.0 software (StatSoft).

Construction of archaeal 16S rRNA gene clone libraries and phylogenetic analysis. The archaeal 16S rRNA gene clone libraries were constructed using the PCR amplicons and a TOPO TA cloning kit (Invitrogen, CA). The cloned inserts that shared a banding profile in restriction fragment length polymorphism analysis with the 2 tetramer restriction enzymes HaeIII and TaqI (New England BioLabs, MA) were grouped into one phylotype, and the sequences of each phylotype were analyzed using an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). The detected archaeal phylotypes were checked for chimeric artifacts using the Pintail program (33), and then the sequence were compared with the sequences in the entire database using a BLAST search. The topology of the phylogenetic tree with 1,000 replicates of bootstrap analysis was constructed with the neighbor-joining method and the Jukes-Cantor correction using the MEGA5 program (34).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences obtained in this study were deposited in the DDBJ/EMBL/GenBank databases under accession numbers KC676291 to KC676307.

RESULTS

Hierarchical primers and specificities. To analyze the methanogens occurring in the methanogenic consortia from wastewater treatment ecosystems using HOPE, we developed 27 16S rRNA-targeted oligonucleotide primers at different taxonomic specificities. The primers were designed with a binding length of 17 to 24 nt and a predicted melting temperature of 47.9°C to 66.8°C (Table 2). To achieve the highest discrimination capability with the fidelity of the *Taq* polymerase, the primers were usually designed with at least 2 mismatches against the nontargeted sequences (17) or a single mismatch at the terminal or penultimate position from the 3' end of the primer (31).

As shown in Fig. 1, one *Archaea*-specific primer, ARC911fm, extended with a G nucleotide could detect most cultivated and uncultivated methanogens. To detect methanogen populations at the order level, four primers, including MSMX859f, MG1197fm, MB1167fm, and MCMT1008fm, specifically extended with a C, C, C, and G nucleotide in *Methanosarcinales*, *Methanomicrobiales*, *Methanobacteriales*, and *Methanococcales*, respectively, were used. For analyzing the acetotrophic, methylotrophic, and hydrogenotrophic groups of methanogens down to the genus level, 22 specific primers were developed. Among them, 9 primers were newly designed and the remaining 13 were adopted from previous studies, with modifications (Table 2). The MS537fm and F3SC979fm primers could be extended with various nucleotides for different groups within *Methanosarcina* and *Methanofollis* (Table 2), respectively, conferring their abilities for multiplex detections per primer. Under the stringent thermal conditions (i.e., a 62°C annealing temperature), all primers could be correctly extended with

TABLE 2 Oligonucleotide primers used for HOPE analysis in this study

Primer	Specificity	Target	Sequence (5'–3') ^a	Length (nt)		Allele	GC (%)	<i>T_m</i> ^d (°C)	C _g ^e (%)	Sty ^f	MTPX	Reference or source
				Primer	Tail							
ARC911fm	Domain	<i>Archaea</i>	(ggat) ₂ TAAAGGAATTGGCGGGG	17	24	G	52.9	53.1	87	20	0, 4	Modified from reference 11
MSMX59f	Order	<i>Methanosarcinales</i>	AGGGAAGCCCGTGAAGCGACG	20	0	C	65.0	62.7	72	174	0, 1a, 1b	Modified from reference 11
MG1197fm	Order	<i>Methanomicrobiales</i>	GTGACGATGCCCCGAATTAT	20	0	C	50.0	55.5	77	20	0, 2a, 2b	Modified from reference 11
MB1167fm	Order	<i>Methanobacteriales</i>	TAAAYWGGAGGAAGAGTGTGA	21	0	C	45.2	53.8	90	12	0, 3	Modified from reference 11
MCMT1008fm	Order	<i>Methanococcales</i>	AGGTTGACGACCTTGGCGWAMGC	23	0	G	60.9	63.8	60	0	0, 4	This study
MX802f	Genus	<i>Methanoseta</i>	GTCTTAGCCGTAAAGCAT	19	0	C	47.4	51.1	78	30	1a	This study
GMHP1258r	Genus	<i>Methanohalophilus</i>	CCGTCACCTTTTACGTGTAGG	20	0	A	50.0	53.1	57	2	1a	17
MMML419r	Genus	<i>Methanomethylovorans</i>	ATGACACAGCCACATAGG	18	0	A	50.0	51.9	60	2	1a	This study
MS537fm	Genus	<i>Methanosarcina</i> spp. I/II ^b	at(aeg) ₂ GGGGCCGAGTGGTATCGT	21	9	G/T	71.4	66.8	75	0	1b	This study
GMLB834fm	Genus	<i>Methanolobus</i>	TGAACGGTGGCACCGTCCCA	21	0	G	61.9	63.9	37	2	1b	Modified from reference 17
GMCO437f	Genus	<i>Methanococcoides</i>	GTCCACATGTGTAAACG	17	0	G	47.1	47.9	86	1	1b	Modified from reference 17
GMLB1225fm	Genus	<i>Methanimitococcus</i>	tgggCAACTACACCGAAAGGTGA	19	4	T	47.4	52.1	8	0	1b	Modified from 17
GMS5261r	Genus	<i>Methanosalsum</i>	GTGGGCTAGCAGGATACCTTG	20	0	C	60.0	57.6	50	0	1b	17
GMCP490fm	Genus	<i>Methanocorpusculum</i>	TGTGAAGAAAGGCGAGGG	18	0	C	55.6	55.0	74	0	2a	Modified from reference 17
GMN829r	Genus	<i>Methanoplanus</i>	(t) ₂ CTGTAGTTACAGGCACACC	20	5	T	55.0	55.1	68	1	2a	Modified from reference 17
SNPP577fm	Genus	<i>Methanoplanus</i> and <i>Methanolactinia</i>	TGAGCAACGACACTGAGAAAG	20	0	T	50.0	54.8	100	1	2a	This study
ML398fm	Genus	<i>Methanolinea</i>	CCGGAAGTCCCGCTAAATTTC	19	0	G	57.9	56.1	84	11	2a	24
F7SC1257f	Genus	<i>Methanospirillum</i>	TCGACACCGAGAGGTGAGG	19	0	A	63.2	58.6	36	4	2b	Modified from reference 17
GMG266f	Genus	<i>Methanogenium</i>	CCCAACAGCCCTGTAATCGA	20	0	T	50.0	55.0	78	6	2b	This study
F3SC979fm	Genus	<i>Methanofolius</i> spp. I/II ^c	(t) ₁ CTCACCGGGTAGACAGCGA	20	4	T/A	65	61.1	94	0	2b	Modified from reference 17
F2SC660fm	Genus	<i>Methanoculleus</i>	(t) ₈ GGAGAGTGAGGGGTACTTCG	21	8	G	61.9	58.7	91	12	2b	Modified from reference 17
GMTB645f	Genus	<i>Methanobrevibacter</i>	TCATGACTCTGAGGTCGG	17	0	G	58.8	53.5	70	1	3	This study
GMSP1129fm	Genus	<i>Methanosphaera</i>	(t) ₄ AACACAATCTTGATTGG	19	4	T	42.1	49.8	12	0	3	This study
GMBS76fm	Genus	<i>Methanobrevibacter</i>	(t) ₁₁ TGAGAAATCGCAGCGGGG	20	11	C	65.0	62.2	84	20	3	This study
GMTT416f	Genus	<i>Methanohernococcus</i>	GCACTGGCTAGGCTTTTCTA	20	0	A	50.0	55.2	60	0	4	Modified from reference 17
GMCT35fm	Genus	<i>Methanococcus</i>	(t) ₁ TCACCGTCGACCCGTTCCAGACA	24	4	A	62.5	65.8	74	0	4	Modified from reference 17
GMCC829f	Genus	<i>Methanocaldococcus</i>	GTGTGCGCTCGGCTTCGGG	19	0	C	73.7	64.1	44	0	4	This study

^a The lowercase letters indicate the tail sequences; M, A/C; W, A/T; Y, C/T.^b *Methanosarcina* group I (ddGTP extended) is *Methanosarcina mazei*, *Methanosarcina barkeri*, *Methanosarcina laciustris*, *Methanosarcina acetivorans*, and *Methanosarcina thermophila*; *Methanosarcina* group II (ddATP extended) is *Methanofolius* group II (ddTTP extended) is *Methanofolius formosanus*.^c *Methanosarcina baltica* and *Methanosarcina semisiae*.^d *T_m*, melting temperature, predicted using OligoAnalyzer version 3.1 (<http://sgidtdna.com/analyser/Applications/OligoAnalyzer/>).^e C_g, fraction of sequences with a perfectly matching primer-binding site in the target group (analyzed using TestProbe version 3.0 with the SILVA SSU r115 database).^f Sty, number of sequences with a perfectly matching primer-binding site outside the target group (analyzed using TestProbe version 3.0 with the SILVA SSU r115 database).

Primer combination for multiplexing. The primers were arranged into 7 sets, with each combination achieving 4 to 6 multiplex detections (Table 2 and Fig. 1). The tube with multiplex primer set 0 (MTPX0) combined an *Archaea*-specific primer, ARC911fm, with 4 order-level primers to detect the methanogens at higher taxonomic ranks, while the other sets (MTPX1 to MTPX4), which each contained one order-level primer and 3 to 5 corresponding genus-specific primers, could detect the targeted

Detection limit. To evaluate the lowest detection limit, we prepared a series of PCR-amplified 16S rRNA gene mixtures with relative abundances ranging from 2.5% to 0.16% *Methanoculleus palmolei* (DSM 4273) in a total template amount of 10 ng to test primers F2SC660fm and MG1197fm, which displayed low fluorescence responses. As shown in Fig. 2a, the results indicated that

FIG 2 Evaluation of the detection limits of HOPE analysis. (a) Fluorescence response in terms of the peak area of the extended primers ddGTP-F2SC660fm and ddCTP-MG1197fm in the HOPE analysis using the tube MTPX2b and known concentrations (0.16% to 2.5%) of the 16S rRNA gene amplified from targeted *Methanoculleus palmolei* (DSM 4273) pooled with nontargeted *Methanocella paludicola* strain SANAE as templates. (b) Relative abundances of order-level methanogens with respect to the total archaeal populations detected using the tube MTPX0 and amplified 16S rRNA gene products from a terephthalate-degrading biofilm (sample 3) as the template. The methanogens affiliated with the orders *Methanobacteriales* targeted by primer MB1167fm and *Methanococcales* targeted by primer MCMT1008fm were not detected. (c) Relative abundances of genus-level methanogens with respect to the total *Methanomicrobiales* populations detected using the tube MTPX2b and varied amounts of amplified 16S rRNA genes from terephthalate-degrading biofilm (sample 3) as the template. The methanogens affiliated with the genera *Methanogenium* targeted by primer GMG266f and *Methanofollis* targeted by primer F3SC979fm were not detected.

the fluorescence intensities of both extended primers decreased linearly ($r^2 > 0.999$), corresponding to a decrease in the amount of DNA of the target *M. palmolei* (DSM 4273), and suggested an approximate abundance of at least 0.16%. We then evaluated the detection limit of the HOPE approach using the PCR-amplified 16S rRNA genes from sample 3. As shown in Fig. 2b, HOPE analysis with primer set MTPX0 could detect methanogens from the orders *Methanosarcinales* and *Methanomicrobiales*, which accounted for $94.3\% \pm 4.5\%$ and $6.6\% \pm 0.1\%$ of the amplified *Archaea* 16S rRNA genes, respectively. Using the tube MTPX2b, as shown in Fig. 2c, the results further indicated the presence of *Methanospirillum* targeted by F7SC1257f under all tested analytical conditions, with a consistent abundance of 10.7% to 11.9% within the amplified *Methanomicrobiales* 16S rRNA genes. *Methanoculleus* targeted by F2SC660fm, with a relative abundance of $3.0\% \pm 0.5\%$ within the amplified *Methanomicrobiales* 16S rRNA genes, was detectable with ≥ 10 ng of the template only. Consequently, the HOPE technique could successfully detect specific methanogens with relative abundances of as low as 0.2% of the

total archaeal 16S rRNA genes amplified from a complex environmental sample. This detection sensitivity is comparable to that of the previous HOPE method developed for the detection of *Bacteroides* and *Prevotella* within the *Bacteroidales* (18–20). To achieve improved detection performance, we applied 15 ng DNA per environmental sample as a template for the HOPE analysis.

Relative abundances of methanogens in consortia analyzed by HOPE. Figure 3 shows the results for the anaerobic sludge samples analyzed using the HOPE approach with 7 sets of primers under the optimized conditions. Among the 27 hierarchical primers, 12 primers exhibited positive primer extension reactions for the archaeal 16S rRNA genes amplified from the 19 sludge samples. Figure 3a displays a color-coded array showing the distribution of the relative abundances of the methanogens detected at different hierarchical levels. At the order level, the results showed that the *Methanosarcinales*-related methanogens detected using the MSMX859f primer occurred in all samples in proportions ranging from 19.5% to 102.9% (standard deviations [SDs], 0.6% to 7.3%) of the PCR-amplified archaeal 16S rRNA genes. The proportion of $>100\%$ detected in some cases was likely the consequence of technical variability. In 9 and 12 samples, respectively, methanogens in the orders *Methanobacteriales* (ddCTP-MB1167fm) and *Methanomicrobiales* (ddCTP-MG1197fm) were detected at relative abundances of 3.3% to 80.7% (SDs, 0.7% to 8.4%) and 3.6% to 52.5% (SDs, 0.1% to 5.9%) in the archaeal 16S rRNA genes amplified from the samples, respectively. The MCMT1008fm primer did not detect the methanogens related to the order *Methanococcales* in any of the sludge samples. The HOPE analysis suggested high detection coverage of methanogens ($>92.8\%$ of amplified *Archaea* 16S rRNA genes) in 14 sludge samples. However, about 12.2% to 22.8% of the archaeal 16S rRNA genes amplified from 5 samples were not covered by the 4 primers. These most likely represented other methanogens or nonmethanogen archaeal archaeons.

Figure 3a further shows the distribution of the relative abundances detected using genus-level primers in relation to their corresponding order-level specificities. It was observed that 8 of the 23 genus-specific primers provided positive dye terminator-extending reactions in the 19 sludge samples. These primers detected 2 or 3 methanogen genera for each order and covered total proportions of 14.3% to 85.7%, 0% to 50.7%, and 0% to 1.2% within the amplified 16S rRNA genes related to the orders *Methanosarcinales*, *Methanomicrobiales*, and *Methanobacteriales*, respectively. Within the order *Methanosarcinales*, the tube MTPX1a detected the MX802f-targeted members of the acetotrophic *Methanosaeta* to be predominant in the 18 sludge samples. Their relative abundance in amplified genes was typically relatively high (60.4% to 90.9% within the *Methanosarcinales*) but could decrease to $<4.3\%$ in the sludge (samples 8 and 12) from a full-scale UASB reactor treating TFT-LCD wastewater. The MTPX1b tube frequently detected *Methanosarcina*, the other acetotrophic methanogen, with the ddGTP-extended primer MS537fm in the samples from full-scale reactors treating TFT-LCD wastewater (samples 8 to 12) and sewage sludge (samples 13 to 19). Their relative abundances in amplified genes ranged from 0.8% to 25.1% within the *Methanosarcinales*. In addition, the methylotrophic *Methanomethylovorans* spp., accounting for a relative abundance of $\leq 71.5\%$ in the amplified *Methanosarcinales* 16S rRNA genes, were also frequently detected in 7 sludge samples using the newly designed primer MML419r.

FIG 3 (a) Color-coded representation of the relative abundances of methanogens detected using HOPE analysis with the 7 primer tubes and amplified 16S rRNA gene products obtained from 19 sludge samples as the templates. (Top) Relative abundances of order-level methanogens with respect to the total archaeal populations analyzed with the tube MTPX0; (bottom) relative abundances of the genus-level methanogens with respect to the corresponding taxonomic orders analyzed using the tubes MTPX1 to MTPX4. The percentages were transformed into a gray scale ranging from 0% (not detected [ND], white) to 100% (black) at intervals of 5%. Abbreviations: U, up-flow anaerobic sludge bed reactor; I, anaerobic fixed film reactor; F, anaerobic fluidized bed reactor; T, fed-batch reactor; A, anaerobic digester; G, granular sludge; B, attached biofilm; S, sludge floc. (b) Spatial distribution of *Methanosarcinales*-related (■) and *Methanobacteriales*-related (□) methanogens with respect to the total archaeal populations; (c) spatial distribution of *Methanosarcina*-related (■), *Methanosaeta*-related (□), and *Methanomethylovorans*-related (■) methanogens with respect to the order *Methanosarcinales*; (d) spatial distribution of *Methanothermobacter*-related methanogens (■) with respect to the order *Methanobacteriales* in a full-scale up-flow anaerobic sludge bed reactor treating TFT-LCD wastewater. The bar represents the standard deviation from triplicate analyses.

Within the order *Methanomicrobiales*, the tubes MTPX2a and MTPX2b could detect the methanogens from 3 genera (*Methanoculleus*, targeted by F2SC660fm; *Methanospirillum*, targeted by F7SC1257f; and *Methanolinea*, targeted by ML398fm) in the samples obtained from the reactors treating sewage sludge (samples 13 to 19) and PTA wastewater-related substrates (samples 1 to 4). According to the obtained data, in the amplified *Methanomicrobiales* 16S rRNA gene products, the *Methanolinea* spp. (10.6% to 101%; SDs, 0.1% to 3.4%) were more abundant than the *Methanoculleus* spp. (1.7% to 27.6%; SDs, <5.0%) and the *Methanospirillum* spp. (3.3% to 11.5%; SDs, 0.2% to 0.6%) in these samples. In addition, 2 genera within the order *Methanobacteriales* could be detected using the tube MTPX3. One was *Methanothermobacter*, targeted by GMTB645f, which occurred with a relative abundance

of 0.9% to 15.9% (SDs, <5.0%) within the amplified *Methanobacteriales* 16S rRNA genes from samples 5, 6, 9, 11, 15, and 19, and the other was *Methanobrevibacter*, targeted by GMB376rm, which occurred with a relative abundance of 0.9% to 7.1% within the amplified *Methanobacteriales* 16S rRNA genes (SDs, 0.1% to 0.2%) from the anaerobic digesters (samples 15 and 19). Our observations on these 5 methanogen genera suggested the occurrence of hydrogenotrophic methanogenesis in the reactors.

Because the methanogen communities in the sludge samples collected from different heights in a full-scale UASB reactor treating TFT-LCD wastewater (samples 8 to 12) showed marked variations, we conducted further analyses. As shown in Fig. 3b, at the order level, *Methanosarcinales*-related methanogens displayed a high abundance within the *Archaea* (59.8% to 73.5% of the am-

plified 16S rRNA genes; SDs, 1.9% to 2.9%) at the bottom of the sludge bed and higher spaces of the reactor, whereas those from the order *Methanobacteriales* were abundant (75.2% to 88.7% of the amplified 16S rRNA genes; SDs, 0.1% to 8.5%) in the center of the reactor (1 to 2 m high). The relative abundance of the *Methanomicrobiales*-related methanogens in the 5 samples tested was below the detection level ($<0.2\%$), suggesting a low density of the population in the ecosystem. At the genus level, as shown in Fig. 3c, the relative abundance of the *Methanomethylovorans* spp. was 60.6% to 71.5% (SDs, 1.4% to 2.2%) within the *Methanosarcinales* 16S rRNA genes or 44.5% to 49.2% within the *Archaea* 16S rRNA genes amplified at the top and bottom of the reactor. Within the amplified *Methanosarcinales* 16S rRNA genes, the *Methanosaeta* spp. were relatively more abundant (61.0% to 72.4%; SDs, 0.6% to 3.5%) than the *Methanomethylovorans* spp. (~18.2%; SDs, 0.6% to 3.2%) and *Methanosarcina* spp. (0.8% to 4.2%; SDs, $<0.1\%$) at reactor heights of 1 to 3 m, and the *Methanobacteriales*-related hydrogenotrophic populations, which accounted for 32.9% to 80.7% (SDs, 0.1% to 8.4%) of the amplified 16S rRNA genes, were highly predominant. However, aside from the *Methanothermobacter* spp., approximately 96.4% of the amplified 16S rRNA genes associated with *Methanobacteriales* were unknown (Fig. 3d).

Archaea 16S rRNA genes) or the unknown *Methanobacteriales*-related populations ($77.6\% \pm 4.3\%$ of the amplified *Archaea* 16S rRNA genes) were dominant in this cluster. Cluster III was represented by samples 13 to 19, which were collected from the 2 full-scale anaerobic digesters on different dates, and was associated with a high abundance of the hydrogenotrophic *Methanolinea* spp. in the archaeal communities ($39.1\% \pm 6.0\%$ of the amplified *Archaea* 16S rRNA genes).

Clone library analysis of the 16S rRNA gene sequences. To validate the HOPE analysis results, 2 archaeal 16S rRNA gene clone libraries were constructed using the DNA from samples 2 and 18. As shown in Fig. 5, all of the sequences obtained in the clone library of sludge sample 2 were phylogenetically associated with the known methanogens. Among them, 92.4%, 4.4%, and 3.8% of the total clones were related to *Methanosarcinales*, *Methanomicrobiales*, and *Methanobacteriales*, respectively. The most predominant methanogens were the acetotrophic *Methanosaeta* (87% of the total clones), followed by the methylotrophic *Methanomethylivorans* (5.4% of the total clones). The hydrogenotrophic methanogens detected included *Methanolinea*, *Methanobacterium*, and *Methanoculleus*, each accounting for <4.3% of the total clones. The other clone library (sample 18) consisted of the sequences retrieved from *Methanosarcinales* (75% of the total clones), *Methanomicrobiales* (18% of the total clones), and uncultured *Euryarchaeota* (6% of the total clones). The most predominant phylotype was the acetotrophic methanogen *Methanosaeta* (75% of the total clones), followed by the 2 hydrogenotrophic populations *Methanospirillum* (9% of the total clones) and *Methanoregula* (3% of the total clones).

The results from the clone libraries were generally in accordance with those from HOPE analysis. Clone library results indicated that the predominant *Methanosaeta* spp. accounted for 75% to 87% of the total clones, and HOPE analysis provided a result of 43% to 64.1% of the amplified *Archaea* 16S rRNA genes. The clone library and HOPE analysis results showed the hydrogenotrophic *Methanolinea* spp. (4.3% versus 6.5%), *Methanoculleus* spp. (1.1% versus 2.6%), and *Methanospirillum* spp. (9% versus 1.5%) and the methyilotrophic *Methanomethylovorans* spp. (5.4% versus 19.4%) to have comparable abundances. However, we observed that certain differences were displayed between the 2 approaches. For example, for sludge sample 18, the *Methanolinea* spp. and *Methanosarcina* spp. with an observable abundance (13.3% to 38.0% of amplified *Archaea* 16S rRNA genes) could be detected using HOPE with primers ML398fm and MS537fm, respectively, whereas the clone library did not retrieve the relevant sequences. The clone library could identify the relative abundance (3.3%) of a *Methanobacterium*-related phylotype (R27-A39) within the *Methanobacteriales*; however, the HOPE analysis using the MB1167fm primer did not detect the methanogens from *Methanobacteriales*.

DISCUSSION

In this study, we successfully developed the HOPE approach to detect the relative abundances of numerous methanogen populations in the pool of archaeal 16S rRNA genes amplified from microbial consortia. After optimizing for specificity, sensitivity, and multiplexing capability, the approach was able to detect known methanogens with a total abundance of up to 77.2% to 107.9% in amplified 16S rRNA genes from total archaeal populations in anaerobic reactors used for the treatment of various industrial

FIG 5 Phylogenetic tree of archaeal 16S rRNA gene sequences retrieved from the sludge of a full-scale UASB reactor treating PTA wastewater (sample 2) and a full-scale anaerobic digester (sample 18). The tree was reconstructed with the 16S rRNA sequences ($>1,200$ bp) of the clones and their close relatives obtained in this study and from the NCBI database on the basis of the neighbor-joining method with 1,000 bootstraps and rooted with *Aquifex pyrophilus*. Black circles at the branch nodes represent the bootstrap values greater than 75%. The bar shows 5% base substitutions. Boldface indicates the cloned representatives and their percentages in the clone library analysis in this study, and those labeled with PTA granule and anaerobic digester (AD) sludge clones were obtained from samples 2 and 18, respectively. The predicated phylogeny is indicated in brackets at the right. TCE, trichloroethylene.

wastewaters and sewage sludge at order and genus specificities. The obtained results strongly suggest that HOPE analysis can improve understanding of the relative abundance of methanogens in complex bioreactor ecosystems.

According to the results obtained by HOPE, the analyzed methanogenic consortia predominantly consisted of methanogen

populations from the order *Methanosarcinales* (ddCTP-extended MSMX859f), *Methanomicrobiales* (ddCTP-extended MG1197fm), and/or *Methanobacteriales* (ddCTP-extended MB1167fm). The acetotrophic and methylophilic methanogens in the order *Methanosarcinales* generally dominated the archaeal communities. The acetotrophic *Methanosaeta* spp. were the most com-

monly detected methanogens in the samples, with a high abundance in sample cluster I (67.1%; SD, 11.6%) and a medium abundance in sample cluster III (38.7%; SD, 4.1%) (Fig. 3 and 4). The results from clone library analysis supported these observations, showing that 87% and 75% of the total clones were associated with the *Methanosaeta* in sludge samples 2 and 18, respectively (Fig. 5). These findings were in high accordance with those from several previous studies (25, 28, 35–37). The dominance of the *Methanosaeta* population indicated its success within the niche for the production of methane from acetate, a common constituent in industrial wastewater (23) as well as a central metabolite resulting from the anaerobic fermentation of substances. In addition to direct use by the acetotrophic methanogens, acetate can be converted by way of a syntrophic interaction between the acetate-utilizing bacteria and hydrogenotrophic methanogens in anaerobic sludge digesters (38, 39). This process might be reflected by our observations of a high abundance of the hydrogenotrophic methanogens and a slightly lower abundance of the acetotrophic methanogens (*Methanosaeta* and *Methanosarcina*) in the anaerobic digesters.

In further analyses, we observed *Methanosaeta* spp. and *Methanosarcina* spp. in sample clusters II and III, suggesting competition for acetate. The *Methanosaeta* population has a high affinity (lower K_s) for acetate and, thus, typically displays a competitive advantage over the *Methanosarcina* spp. at low acetate concentrations (40). However, *Methanosarcina* could produce methane using all three known methanogenesis pathways (2) and would be more competitive than *Methanosaeta* in high acetate concentrations (41) and high temperatures (55 to 60°C) (17). In this study, the *Methanosarcina* population showed an approximately 16.5% higher abundance than *Methanosaeta* in sample 12, which was collected from a sampling port in proximity to the effluent of the UASB reactor. This observation suggests that it might confer high competitive capabilities for using substrates such as acetate and methylated amines under more oxidizing redox conditions.

Although several studies have detected 16S rRNA sequences related to the *Methanomethylovorans*-like archaeons in freshwater sediments (42) as well as in anaerobic reactors (9, 43, 44) using DGGE, cloning/sequencing, and SSCP techniques, their quantity is poorly understood. To our knowledge, no previous study has designed probes/primers for quantifying the relative abundance of *Methanomethylovorans* spp. in environmental samples (17). In this study, we were able to effectively evaluate the relative abundance of *Methanomethylovorans* 16S rRNA gene sequences amplified from the samples of 4 reactors using the HOPE technique and a newly designed primer, MML419r. The finding indicated an important role of *Methanomethylovorans* in the ecosystem of anaerobic reactors and revealed that for the anaerobic treatment of industrial wastewater, methylotrophic methanogenesis contributes a proportion of the methane formed. This process was particularly prominent in the treatment of TFT-LCD wastewater (i.e., cluster II sludge samples), because the principal wastewater constituent was methylated compounds.

The known members within the orders *Methanomicrobiales* and *Methanobacteriales* are hydrogenotrophic. These groups of methanogens were commonly detected inside the anaerobic reactors, and their abundances could also be clearly differentiated among the 3 clusters of samples. For example, the amplified 16S rRNA genes of hydrogenotrophic methanogens from the order *Methanomicrobiales* exhibited a high abundance (34.2% to 48.5%)

in sample cluster III (samples 13 to 19) and a medium-level abundance (~7.0%) in sample cluster I (samples 1 to 7) but were undetectable in sample cluster II. Closer examination at the genus level revealed the prevalence of 3 genera. The *Methanospirillum* spp. and *Methanoculleus* spp. are important hydrogen scavengers in anaerobic digesters (9, 45) and constituted 0.2% to 1.6% and 0.1% to 3.2% of the amplified *Archaea* 16S rRNA genes, respectively, in the reactors treating sewage sludge, terephthalate, and PTA wastewater. Imachi et al. recently identified the *Methanolinea* genus, obtaining the first species, *Methanolinea tarda*, from an anaerobic digester (46). The HOPE analysis findings suggested that the *Methanolinea* spp. were relatively abundant (31.6% to 45.0% of the amplified *Archaea* 16S rRNA genes) in anaerobic digesters (cluster III sludge samples), and their abundances could be distinctively discriminated from other sources of sludge samples (0.7% to 6.5% of the amplified *Archaea* 16S rRNA genes, clusters I and II). In addition to reactor ecosystems, this methanogenic archaeon was present in natural environments (47–49). Its relatively extensive distribution suggests that this novel archaeon plays an important role in various methanogenic ecosystems.

In this study, the HOPE method also detected members in the order *Methanobacteriales*, hydrogenotrophic methanogens from the genera *Methanobrevibacter* and *Methanothermobacter* that have frequently been observed in methanogenic processes (9, 45, 50), using 2 new primers (GMB376rm and GMTB645f, respectively). Their individual abundances were usually lower than 1.2% in the amplified *Archaea* 16S rRNA genes, with no distinct distribution pattern among the 3 sample clusters (Fig. 3 and 4). In a previous study with the RNase H method, it was found that the 2 populations were active and represented the major groups within the *Methanobacteriaceae* in the reactors treating sugar- and clear-liquor-processing wastewaters (17). However, among the 6 samples in this study, the *Methanobrevibacter* spp. and *Methanothermobacter* spp. accounted for <19.2% of the amplified *Methanobacteriales* 16S rRNA genes, suggesting the presence of a large proportion of other methanogens within the same order. As shown in Fig. 4, the high predominance (~80.7% of the amplified *Archaea* 16S rRNA genes) of the unknown *Methanobacteriales*-related groups suggested their significance in the full-scale UASB reactor treating TFT-LCD wastewater. The low percentages covered by the genus-specific primers within the order *Methanobacteriales* were likely attributed to a lack of primers to detect the genus *Methanobacterium*, which was actually detected using the cloning library (Fig. 5), and/or the mysterious groups. It should be particularly mentioned that no primers with a good coverage for the genus *Methanobacterium* could be developed. We actually excluded a primer, GMBA755, designed by Narihiro and coworkers (17) from the present work, because the specificity would be compromised in the HOPE analysis. To solve this, several primers with individual specificities below the genus level can be designed to collectively achieve a better coverage of various groups within the genus *Methanobacterium* in the future, and HOPE can easily detect a target at the species level (18). With the advantages in detection sensitivity and throughput, HOPE can complement other available molecular methods, such as the cloning library method, to further study the mysterious groups.

Our study results further demonstrated the ability of the HOPE approach to identify the associations between reactor operations and methanogen diversity. As shown in Fig. 3, the population

structures of the methanogens observed in sludge samples 13 to 19 were relatively constant, suggesting the stable dynamics of the archaeal community in the 2 anaerobic digesters within the sampling periods. In contrast, as shown in Fig. 3b to d, the abundances of the methanogen populations in a full-scale UASB reactor treating TFT-LCD wastewater showed marked changes along the height of the reactor. Similarly, in a full-scale AFB reactor, the *Methanomethylovorans* spp. and *Methanomicrobiales*-related populations, including *Methanoculleus* spp., were detected in the biofilm sampled at a 3-m height in the reactor but were undetectable in the biofilm sampled at the top of the reactor (19 m). It is likely that this differential distribution pattern reflects differences in environmental conditions, which might be associated with the vertical configuration of the reactors, operational conditions (organic loading, up-flow velocity, and effluent recirculation), and wastewater characteristics.

As demonstrated, HOPE represents a hierarchical approach to analyze the entire set of PCR-amplified products from the archaeal community. Using the optimized conditions obtained in this study, the method is considerably straightforward for estimating the relative abundance of known methanogen targets at a sensitivity corresponding to 0.2% of the amplified 16S rRNA genes (Fig. 2). The reliability of the HOPE results obtained in this study was validated using a clone library approach (Fig. 5), in which the abundance of a specific phylotype is dependent on the ultimate number of clones analyzed. Similar to the cloning library, the use of HOPE to determine the relative abundance of PCR-amplified products can be subject to PCR biases (51), while it is occasionally difficult to obtain a single primer with specificity for a diverse group. These are the likely causes of the dissimilarities in the results between the HOPE method and cloning library analysis. Therefore, special care, such as collection of DNA products from sludge samples at the exponential phase of PCR amplification (19) and careful evaluation of primer specificity (31), should be taken to achieve the accuracy of relative abundance quantitation.

It is clear that through comparison of the analytical throughput in a single reaction, the HOPE method can be in a niche between the low-throughput methods, such as FISH, qPCR, and the RNase H method, and the high-throughput microarray platform method. The developed HOPE assay can detect 22 targeted methanogens at different taxonomic levels (513 reactions = 27 primers $\times$ 19 samples) within 8 to 9 h, a time which is comparable to that needed for the RNase H method (17). To have a complete set of methanogen targets, more primers should be designed for the recently recognized *Methanocellales* order and 11 methanogen genera not yet included in this study. The total number of methanogen targets can be flexibly increased by adding more HOPE reactions or by adding a primer(s) to individual reactions (Fig. 1). When combined with a qPCR to analyze the absolute quantity of total archaeal 16S rRNA genes, the HOPE analysis can be more quantitative for methanogens and has high applicability to the samples from natural environments.

In summary, the HOPE method with a large number of primers can successfully determine the relative abundances of known methanogens in amplified 16S rRNA gene products at order and genus specificities in a rapid and high-throughput manner. The overall results provide insight into the diversity, temporal dynamics, and spatial distributions of the acetotrophic, hydrogenotrophic, and methylotrophic methanogens and suggest that in addition to the acetotrophic *Methanosaeta*, the recently identified

hydrogenotrophic *Methanolinea* and methylotrophic *Methanomethylovorans* play important roles inside anaerobic reactors treating industrial wastewater and sewage sludge. Considering its technical simplicity and robust multiplexing capabilities, the HOPE approach can be used as a platform for the routine monitoring of methanogen populations for improved management of process operations and could facilitate relevant studies of the methanogen populations in various methanogenic ecosystems, especially whenever analysis of a large number of samples is needed.

ACKNOWLEDGMENTS

We thank I-An Tsai and Tsuyoshi Yamaguchi for their experimental assistance and Hiroyuki Imachi for kindly providing the reference strain. The comments of W.-T. Liu and H. Tamaki were deeply appreciated, too.

This research was supported by a grant (NSC97-2221-E006-038-MY3) from Taiwan's National Science Council.

REFERENCES

- Lettinga G, Field J, vanLier J, Zeeman G, Pol LWH. 1997. Advanced anaerobic wastewater treatment in the near future. *Water Sci. Technol.* 35:5–12.
- Liu Y, Whitman WB. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann. N. Y. Acad. Sci.* 1125:171–189.
- Weijers JW, Schouten S, van der Linden M, van Geel B, Damste JS. 2004. Water table related variations in the abundance of intact archaeal membrane lipids in a Swedish peat bog. *FEMS Microbiol. Lett.* 239:51–56.
- Neu TR, Kuhllicke U, Lawrence JR. 2002. Assessment of fluorochromes for two-photon laser scanning microscopy of biofilms. *Appl. Environ. Microbiol.* 68:901–909.
- Talbot G, Topp E, Palin MF, Masse DI. 2008. Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. *Water Res.* 42:513–537.
- Steinberg LM, Regan JM. 2008. Phylogenetic comparison of the methanogenic communities from an acidic, oligotrophic fen and an anaerobic digester treating municipal wastewater sludge. *Appl. Environ. Microbiol.* 74:6663–6671.
- Akarsubasi AT, Ince O, Kirdar B, Oz NA, Orhon D, Curtis TP, Head IM, Ince BK. 2005. Effect of wastewater composition on archaeal population diversity. *Water Res.* 39:1576–1584.
- Lueders T, Friedrich M. 2000. Archaeal population dynamics during sequential reduction processes in rice field soil. *Appl. Environ. Microbiol.* 66:2732–2742.
- Leclerc M, Delgenes JP, Godon JJ. 2004. Diversity of the archaeal community in 44 anaerobic digesters as determined by single strand conformation polymorphism analysis and 16S rDNA sequencing. *Environ. Microbiol.* 6:809–819.
- Gagnon N, Barret M, Topp E, Kalmokoff M, Masse D, Masse L, Talbot G. 2011. A novel fingerprint method to assess the diversity of methanogens in microbial systems. *FEMS Microbiol. Lett.* 325:115–122.
- Raskin L, Stromley JM, Rittmann BE, Stahl DA. 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.* 60:1232–1240.
- Raskin L, Poulsen LK, Noguera DR, Rittmann BE, Stahl DA. 1994. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* 60:1241–1248.
- Zheng D, Raskin L. 2000. Quantification of *Methanosaeta* species in anaerobic bioreactors using genus- and species-specific hybridization probes. *Microb. Ecol.* 39:246–262.
- Steinberg LM, Regan JM. 2009. mcrA-targeted real-time quantitative PCR method to examine methanogen communities. *Appl. Environ. Microbiol.* 75:4435–4442.
- Yu Y, Lee C, Kim J, Hwang S. 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. *Biotechnol. Bioeng.* 89:670–679.
- Narihiro T, Sekiguchi Y. 2011. Oligonucleotide primers, probes and molecular methods for the environmental monitoring of methanogenic archaea. *Microb. Biotechnol.* 4:585–602.
- Narihiro T, Terada T, Ohashi A, Wu JH, Liu WT, Araki N, Kamagata

- Y, Nakamura K, Sekiguchi Y. 2009. Quantitative detection of culturable methanogenic archaea abundance in anaerobic treatment systems using the sequence-specific rRNA cleavage method. *ISME J.* 3:522–535.
18. Wu JH, Liu WT. 2007. Quantitative multiplexing analysis of PCR-amplified ribosomal RNA genes by hierarchical oligonucleotide primer extension reaction. *Nucleic Acids Res.* 35:e82. doi:10.1093/nar/gkm413.
19. Hong PY, Wu JH, Liu WT. 2008. Relative abundance of *Bacteroides* spp. in stools and wastewaters as determined by hierarchical oligonucleotide primer extension. *Appl. Environ. Microbiol.* 74:2882–2893.
20. Hong PY, Wu JH, Liu WT. 2009. A high-throughput and quantitative hierarchical oligonucleotide primer extension (HOPE)-based approach to identify sources of faecal contamination in water bodies. *Environ. Microbiol.* 11:1672–1681.
21. Wu JH, Hsu MH, Hung CH, Tseng IC, Lin TF. 2010. Development of a hierarchical oligonucleotide primer extension assay for the qualitative and quantitative analysis of *Cylindrospermopsis raciborskii* subspecies in freshwater. *Microbes Environ.* 25:103–110.
22. Nikolausz M, Chatzinotas A, Palatinszky M, Imfeld G, Martinez P, Kastner M. 2008. Single-nucleotide primer extension assay for detection and sequence typing of “*Dehalococcoides*” spp. *Appl. Environ. Microbiol.* 74:300–304.
23. Cheng SS, Ho CY, Wu JH. 1997. Pilot study of UASB process treating PTA manufacturing wastewater. *Water Sci. Technol.* 36:73–82.
24. Wu JH, Wu FY, Chuang HP, Chen WY, Huang HJ, Chen SH, Liuc WT. 2013. Community and proteomic analysis of methanogenic consortia degrading terephthalate. *Appl. Environ. Microbiol.* 79:105–112.
25. Chen CL, Wu JH, Tseng IC, Liang TM, Liu WT. 2009. Characterization of active microbes in a full-scale anaerobic fluidized bed reactor treating phenolic wastewater. *Microbes Environ.* 24:144–153.
26. Hu TH, Whang LM, Liu PW, Hung YC, Chen HW, Lin LB, Chen CF, Chen SK, Hsu SF, Shen W, Fu R, Hsu R. 2012. Biological treatment of TMAH (tetra-methyl ammonium hydroxide) in a full-scale TFT-LCD wastewater treatment plant. *Bioresour. Technol.* 113:303–310.
27. Rasmussen JP, Barbez PH, Burgoyne LA, Saint CP. 2008. Rapid preparation of cyanobacterial DNA for real-time PCR analysis. *Lett. Appl. Microbiol.* 46:14–19.
28. Wu JH, Liu WT, Tseng IC, Cheng SS. 2001. Characterization of microbial consortia in a terephthalate-degrading anaerobic granular sludge system. *Microbiology* 147:373–382.
29. Embley TM, Finlay BJ, Thomas RH, Dyal PL. 1992. The use of rRNA sequences and fluorescent probes to investigate the phylogenetic positions of the anaerobic ciliate *Metopus palaeformis* and its archaeobacterial endosymbiont. *J. Gen. Microbiol.* 138:1479–1487.
30. Grosskopf R, Janssen PH, Liesack W. 1998. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl. Environ. Microbiol.* 64:960–969.
31. Wu JH, Hong PY, Liu WT. 2009. Quantitative effects of position and type of single mismatch on single base primer extension. *J. Microbiol. Methods* 77:267–275.
32. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH. 2004. ARB: a software environment for sequence data. *Nucleic Acids Res.* 32:1363–1371.
33. Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ. 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Appl. Environ. Microbiol.* 71:7724–7736.
34. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28:2731–2739.
35. Chen CL, Macarie H, Ramirez I, Olmos A, Ong SL, Monroy O, Liu WT. 2004. Microbial community structure in a thermophilic anaerobic hybrid reactor degrading terephthalate. *Microbiology* 150:3429–3440.
36. Chen CL, Wu JH, Liu WT. 2008. Identification of important microbial populations in the mesophilic and thermophilic phenol-degrading methanogenic consortia. *Water Res.* 42:1963–1976.
37. Perkins SD, Scalfone NB, Angenent LT. 2011. Comparative 16S rRNA gene surveys of granular sludge from three upflow anaerobic bioreactors treating purified terephthalic acid (PTA) wastewater. *Water Sci. Technol.* 64:1406–1412.
38. Hattori S. 2008. Syntrophic acetate-oxidizing microbes in methanogenic environments. *Microbes Environ.* 23:118–127.
39. Ito T, Yoshiguchi K, Ariesyady HD, Okabe S. 2011. Identification of a novel acetate-utilizing bacterium belonging to *Synergistes* group 4 in anaerobic digester sludge. *ISME J.* 5:1844–1856.
40. Min H, Zinder SH. 1989. Kinetics of acetate utilization by two thermophilic acetotrophic methanogens: *Methanosarcina* sp. strain CALS-1 and *Methanotherox* sp. strain CALS-1. *Appl. Environ. Microbiol.* 55:488–491.
41. Conklin A, Stensel HD, Ferguson J. 2006. Growth kinetics and competition between *Methanosarcina* and *Methanosaeta* in mesophilic anaerobic digestion. *Water Environ. Res.* 78:486–496.
42. Lomans BP, Maas R, Luderer R, Op den Camp HJ, Pol A, van der Drift C, Vogels GD. 1999. Isolation and characterization of *Methanomethylovorans hollandica* gen. nov., sp. nov., isolated from freshwater sediment, a methylotrophic methanogen able to grow on dimethyl sulfide and methanethiol. *Appl. Environ. Microbiol.* 65:3641–3650.
43. de Bok FA, van Leerdam RC, Lomans BP, Smidt H, Lens PN, Janssen AJ, Stams AJ. 2006. Degradation of methanethiol by methylotrophic methanogenic archaea in a lab-scale upflow anaerobic sludge blanket reactor. *Appl. Environ. Microbiol.* 72:7540–7547.
44. Rodriguez E, Lopes A, Fdz-Polanco M, Stams AJ, Garcia-Encina PA. 2012. Molecular analysis of the biomass of a fluidized bed reactor treating synthetic vinasse at anaerobic and micro-aerobic conditions. *Appl. Microbiol. Biotechnol.* 93:2181–2191.
45. Franke-Whittle IH, Goberna M, Pfister V, Insam H. 2009. Design and development of the ANAEROCHIP microarray for investigation of methanogenic communities. *J. Microbiol. Methods* 79:279–288.
46. Imachi H, Sakai S, Sekiguchi Y, Hanada S, Kamagata Y, Ohashi A, Harada H. 2008. *Methanolinea tarda* gen. nov., sp. nov., a methane-producing archaeon isolated from a methanogenic digester sludge. *Int. J. Syst. Evol. Microbiol.* 58:294–301.
47. Sakai S, Ehara M, Tseng IC, Yamaguchi T, Brauer SL, Cadillo-Quiroz H, Zinder SH, Imachi H. 2012. *Methanolinea mesophila* sp. nov., a hydrogenotrophic methanogen isolated from rice field soil, and proposal of the archaeal family *Methanoregulaceae* fam. nov. within the order *Methanomicrobiales*. *Int. J. Syst. Evol. Microbiol.* 62:1389–1395.
48. Singh DN, Kumar A, Sarbhai MP, Tripathi AK. 2012. Cultivation-independent analysis of archaeal and bacterial communities of the formation water in an Indian coal bed to enhance biotransformation of coal into methane. *Appl. Microbiol. Biotechnol.* 93:1337–1350.
49. Tang YQ, Li Y, Zhao JY, Chi CQ, Huang LX, Dong HP, Wu XL. 2012. Microbial communities in long-term, water-flooded petroleum reservoirs with different in situ temperatures in the Huabei Oilfield, China. *PLoS One* 7:e33535. doi:10.1371/journal.pone.0033535.
50. Zheng D, Angenent LT, Raskin L. 2006. Monitoring granule formation in anaerobic upflow bioreactors using oligonucleotide hybridization probes. *Biotechnol. Bioeng.* 94:458–472.
51. Forney LJ, Zhou X, Brown CJ. 2004. Molecular microbial ecology: land of the one-eyed king. *Curr. Opin. Microbiol.* 7:210–220.